Chemotactic Peptide Analogues

Synthesis and Chemotactic Activity of *N*-Formyl-Met-Leu-Phe Analogues Containing (*S*)-Phenylalaninol Derivatives

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Key Words: *N-formyl peptide analogues, human neutrophils, chemotaxis, superoxide anion production, lysozyme release*

Summary

The synthesis and the biological activity towards human neutrophils of some *N*-formyl-Met-Leu-Phe-OMe analogues containing (*S*)-phenylalaninol (Pheol) or its derivatives in place of the native phenylalanine are reported. While the analogue containing Pheol (**4**) was found to be devoid of significant biological activity, its esters **3** and **5**, although inactive as chemoattractants, are able to strongly stimulate superoxide production and are active with a lower efficacy in the lysozyme release.

Introduction

Chemotactic *N*-formylpeptides are involved in defence mechanisms against bacterial infections. In addition to directional neutrophil migration from blood to sites of infection, the interaction of chemotactic factors with specific membrane receptors stimulates several biological functions as superoxide anion generation and lysosomal enzyme release. *N*-Formyl-methionyl-leucyl-phenylalanine methyl ester (*N*-formyl-Met-Leu-Phe-OMe) is the reference compound in the family of the formylpeptides and a large number of analogues of this ligand have been examined in order to correlate structure-activity data and determine the structural features optimal for the biological activity. It has been emphasized the crucial requirement for the formylmethionine and phenylalanine at position 1 and 3 of the formyltripeptide, respectively, both for the binding and the biological activity [1–3].

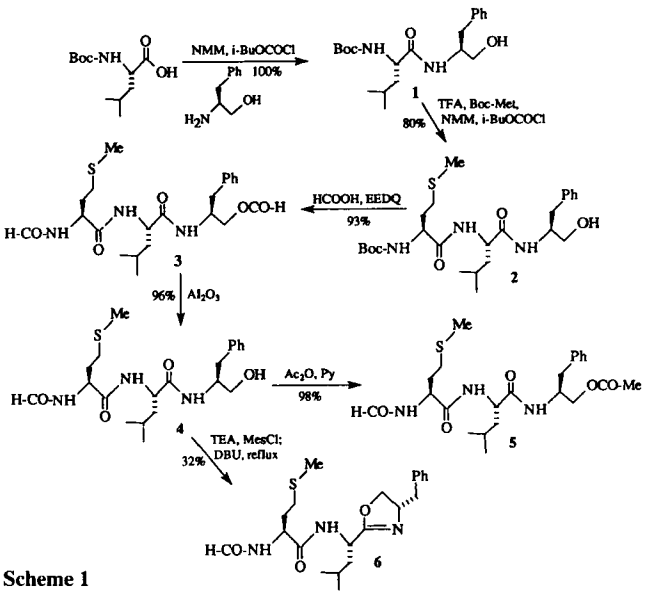
We have recently discussed the loss of biological activity towards human neutrophils shown by two analogues containing (*Z*)-2,3-didehydrophenylalanine (Δ^zPhe) in place of phenylalanine [4]. Other papers deal with modifications at the *C*-terminal Phe residue in *N*-formyl-Met-Leu-Phe-OMe [5,6]. In order to gain further information on the role exerted by the phenylalanine carbonyl group in the binding interaction with the receptors, we report here synthesis and biological activity of some *N*-formyl-Met-Leu-Phe-OMe analogues (**3–6**) in which (*S*)-phenylalaninol (Pheol) or its derivatives replace the native Phe residue. The presence of β-amino alcohols in bioactive peptides at the *C*-terminus such as in peptaibols [7], an important family of natural antibiotics, in enzyme inhibitors [8], and in enkephalin analogues [9] is well documented. In the analogue**4** the CH_2OH group replaces the *C*-terminal ester function which is restored in formate **3** and in acetate **5**; a *C*-terminal oxazoline ring characterizes the compound **6**. Our main purpose is to verify if a H-bond interaction involving the free OH group of **4** may play a role in the binding with an acceptor site of the receptor.

Results and Discussion

Chemistry

The synthesis of the *N*-formyl derivatives **3-6** was performed according to Scheme 1.

The key intermediate **2** was obtained by stepwise elongation using the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM). Deprotection of the *N*-Boc protected primary alcohol dipeptide **1** was performed by treatment with trifluoroacetic acid (TFA). Treatment of **2** with formic acid followed by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) gave the diformyl derivative **3**. Selective hydrolysis of **3** to yield the hydroxy derivative **4** was achieved by stirring in tetrahydrofuran (THF) with water deactivated neutral alumina[10]. Acetylation of **4** with acetic anhydride in the presence of pyridine (Py), afforded the *N*-formyl acetate **5**. Finally, treatment of the THF solution of **4** with mesyl chloride (MesCl) and triethylamine (TEA), followed by refluxing in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) afforded the 2-oxazoline **6**.



Scheme 1

  0365-6233/95/0909-0673 $5.00 + .25/0

Biological Activity

The biological activity of compounds **3–6** has been determined on human neutrophils and compared to that of the parent *N*-formyl-Met-Leu-Phe-OMe. The directed migration (chemotaxis), superoxide anion production, and lysozyme release have been measured. In the adopted conditions all the modified peptides are unable to activate the chemotaxis (Fig. 1A), while are active as superoxide anion generating agents, although with a different efficacy (Fig. 1B). In particular the acetate **5** exhibits an activity significantly higher ($p < 0.05$) than the parent at physiological concentration of 10^{-6} M (44 and 56 nmoles of O_2^- are produced by *N*-formyl-Met-Leu-Phe-OMe and **5**, respectively) and reaches the maximum activity at 2×10^{-6} M. The formate **3** shows an activity similar to that of the parent at 10^{-6} M, being maximally effective at 2×10^{-6} M. The oxazoline derivative **6** is less active than the parent acting only at high concentrations (2×10^{-6}–10^{-5}); the Pheol containing analogue **4** seems to retain some activity only at very high concentrations. Finally a lower efficacy as secretagogue agents, when compared with *N*-formyl-Met-Leu-Phe-OMe, is exhibited by all derivatives at optimal concentrations 10^{-6}–10^{-5} M (Fig. 1C), and the order of potency is **5** > **3** > **6** > **4**.

Conclusion

From the above reported data it can be pointed out that the primary alcohol tripeptide **4** is practically devoid of biological activity while its esters **3** and **5**, although inactive as chemoattractants, are able to strongly stimulate the superoxide production and are active with a lower efficacy in the lysozyme release. These results agree with the findings of Freer *et al.*[2] on the crucial role of the Phe carbonyl for a good biological activity, and indicate that the OH group of the primary alcohol tripeptide **4** does not fulfill the binding requirements to the appropriate area of the receptor. The hydrogen bond interaction of the Phe C=O with the receptor, proposed by the above workers, is lacking in the case of the decarboxy analogue**4**, while it may be operative in the esters **3** and **5** which contain a C=O ester function at the third residue; it should be noted that in these two latter compounds the ester function, although present, is translated by two bonds and its direction is reversed [–O–C(=O)– vs. –C(=O)–O–] as compared to the parent compound. Our results are in accordance with those obtained by studying the amide derivatives of *N*-formyl-Met-Leu-Phe[2,11]; these analogues are found to be even more potent than the parent compound, whereas *N*-formyl-Met-Leu-β-phenylethylamine [2], in which the carboxyl was eliminated, shares the lack of chemotactic activity with the phenylalaninol derivative **4.**

The oxazoline O–C=N group of **6** which is less active than **3** and **5** is probably less effective, in comparison with the carbonyl, for the H-bond interaction. Finally it can be noted that whereas the parent *N*-formyl-Met-Leu-Phe-OMe is able to elicit all the tested biological responses, both **3** and **5** are selective in the production of the superoxide anion and release of lysozyme. An analogous behaviour was already observed by us in the case of a previously described *N*-formyltripeptide containing an α,β-unsaturated residue at the central position [12].

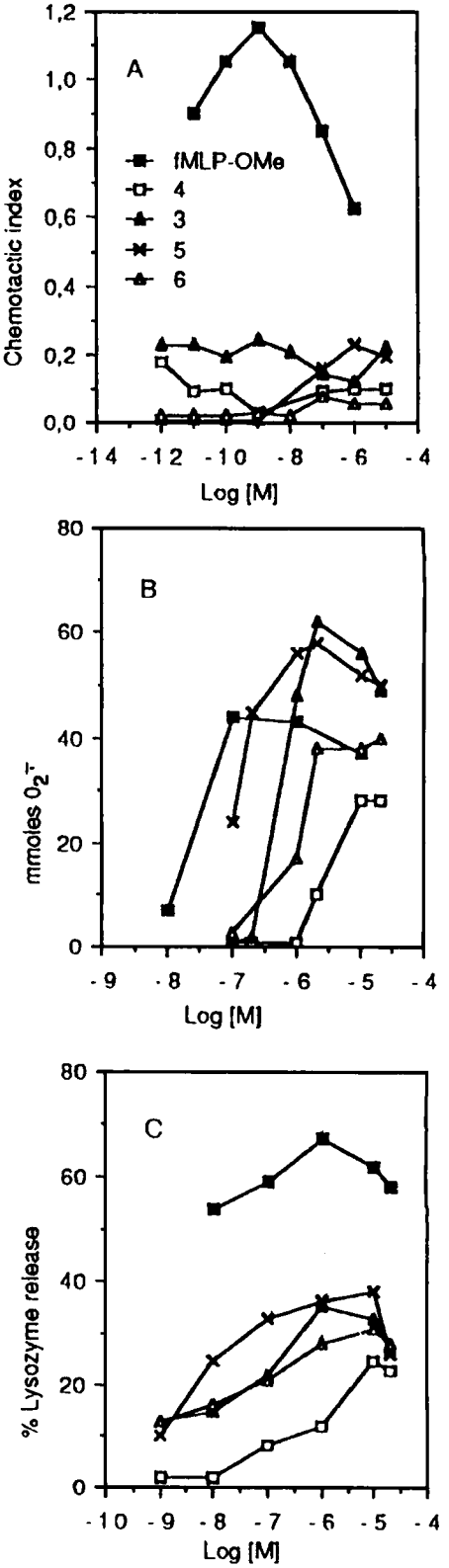


Figure 1. Biological activity of fMLP-OMe and its analogues **3–6** towards human neutrophils. (A) Chemotactic activity. The points are the mean of six separate experiments done in duplicate. Standard errors are in the 0.02–0.09 chemotactic index range. (B) Superoxide anion production. The points are the mean of six separate experiments done in duplicate. Standard errors are in 0.1–4 nmoles O_2^- range. (C) Release of neutrophil granule enzymes evaluated by determining lysozyme activity. The points are the mean of five separate experiments done in duplicate. Standard errors are in 1–6% range.

Experimental Part

Chemistry

Melting points: Kofler hot stage apparatus, uncorrected.– Optical rotations: Schmidt-Haensch Polartronic D polarimeter, 1 dm cell, 20°C.– IR spectra: Perkin-Elmer 983 spectrophotometer.– ^{1}H-NMR spectra: Varian XL-300 spectrometer (tetramethylsilane as int. standard).– Column chromatographies: Merck silica gel 60 (230–400 mesh) (1:30).– TLC and PLC: Merck 60 F_{254} silica gel plates.– Drying agent: Na_2SO_4.– Light petroleum: 40–60 °C bp. fraction.– *N*-formyl-Met-Leu-Phe-OMe was synthesized according to ref. [13].

The abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulphoxide.

Boc-Leu-Pheol (1)

Isobutyl chloroformate 95% (0.83 ml, 6 mmoles) was added at –15 °C to a stirred solution of Boc-Leu-OH·H_2O (1.496 g, 6 mmoles) and NMM (0.7 ml, 7.2 mmoles) in dry CH_2Cl_2 (29 ml). The temp. was kept at –15 °C for 10 min, then a solution of an equimolar amount (0.907 g, 6 mmoles) of Pheol in dry CH_2Cl_2 (22 ml) was added. The mixture was stirred at –15 °C for 15 min and then at room temp. overnight. EtOAc was added in excess and the org. layers were washed with 5% aqueous $KHSO_4$, brine, saturated aqueous $NaHCO_3$, and brine. The collected org. phases were dried and evaporated under reduced pressure to give pure title compound **1** (2.189 g, 100%), mp. 123–124 °C (diethyl ether).– $[\alpha]_D$ –51° (*c* 1.0, $CHCl_3$).– R_F 0.43 in CH_2Cl_2/EtOAc (1:1).– IR (KBr): 3347; 3307; 1681; 1658; 1523 cm^{-1}.– ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.87 (6H, d, *J* = 7 Hz, $CH(CH_3)_2$), 1.35–1.75 (12H, m, $(CH_3)_3C$ (s at 1.42) superimposed on Leu β-CH_2 and γ-CH), 2.88 (2H, d, *J* = 7 Hz, CH-CH_2-Ph), 3.61 (3H, apparent d, CH_2OH), 4.13 (2H, m, Leu α-CH and CH-CH_2OH), 5.27 (1H, br d, NHCOO), 6.88 (1H, d, *J* = 8.5 Hz, Pheol NH), 7.28 (5H, s, aromatic).– $C_{20}H_{32}N_2O_4$ (364.5) Calcd. C 65.9 H 8.85 N 7.7 Found C 65.9 H 9.20 N 7.7.

Boc-Met-Leu-Pheol (2)

Boc-Leu-Pheol (0.182 g, 0.5 mmol) was dissolved in a mixture of dry $CHCl_3$ (0.6 ml) and TFA (0.3 ml) and stirred at room temp. for 5 h. The organic solvent was removed under reduced pressure and the oily H-Leu-Pheol·TFA was dried under vacuum overnight. Isobutyl chloroformate 95% (0.07 ml, 0.5 mmol) was added at –15 °C to stirred solution of Boc-Met-OH (0.125 g, 0.5 mmol) and NMM (0.07 ml, 0.6 mmol) in dry CH_2Cl_2 (2.4 ml). The temp. was kept at –15 °C for 10 min, then a solution of H-Leu-Pheol·TFA (0.5 mmol) in dry CH_2Cl_2 (2.4 ml) and dry DMF (0.7 ml), neutralized with NMM, was added. Stirring and work up, performed as described for compound **1**, afforded a residue (0.271 g) which was chromatographed on a silica column. Elution with CH_2Cl_2/EtOAc (7:3 and 1:1) gave pure Boc-Met-Leu-Pheol (**2**) (0.198 g, 80%), mp. 133–134 °C (ether/light petroleum).– $[\alpha]_D$ –61° (*c* 1.0, $CHCl_3$).– R_F 0.33 in CH_2Cl_2/EtOAc (1:1).– IR (KBr): 3291; 1686; 1643; 1524 cm^{-1}.– ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.87 (6H, d, *J* = 7 Hz, $CH(CH_3)_2$), 1.35–1.75 (12H, m, $(CH_3)_3C$ (s at 1.43) superimposed on Leu β-CH_2 and γ-CH), 1.80–2.07 (2H, m, Met β-CH_2), 2.06 (3H, s, SCH_3), 2.51 (2H, t, *J* = 7.5 Hz, CH_2S), 2.87 (2H, d, *J* = 8 Hz, CH-CH_2-Ph), 3.61 (3H, poorly resolved d, CH_2OH), 3.97–4.60 (3H, m, Leu α-CH, Met α-CH, and CH-CH_2OH), 5.68 (1H, d, *J* = 8 Hz, NHCOO), 6.98–7.40 (7H, m, aromatic, Pheol NH, and Leu NH).– $C_{25}H_{41}N_3O_5S$ (495.7) Calcd. C 60.6 H 8.34 N 8.5 Found C 60.4 H 8.74 N 8.3.

HCO-Met-Leu-Pheol formate (3)

A solution of **2** (0.716 g, 1.445 mmoles) in HCOOH (8.7 ml) was stirred at room temp. for one day and then evaporated under vacuum. The oily residue was dissolved in dry DMF (8.7 ml) and EEDQ 97% (0.442 g, 1.734 mmoles) was added. The solution was stirred at room temp. for 20 h. Evaporation under reduced pressure afforded a residue which was dissolved in MeOH, and the product was precipitated by light petroleum. Washing with dry ether afforded pure title formate **3** (0.606 g, 93%), mp. 175–176 °C.– $[\alpha]_D$ –48° (*c* 0.5, MeOH).– R_F 0.23 in CH_2Cl_2/EtOAc (1:1).– IR (KBr): 3280; 1723; 1665; 1631; 1547 cm^{-1}.– ^{1}H-NMR ([D_6]DMSO): δ (ppm) = 0.81 and 0.86 (6H, two d, *J* = 6.5 Hz, $(CH_3)_2CH$), 1.37 (2H, m, Leu β-CH_2), 1.51 (1H, m, Leu γ-CH), 1.66–1.91 (2H, m, Met β-CH_2), 2.02 (3H, s, SCH_3), 2.40 (2H, t, *J* = 7.5 Hz, CH_2S), 2.78 (2H, sharp m, Ph-CH_2-CH), 3.95 (1H, m, 1 H of CH_2OCO), 4.07–4.31 (3H, m, 1 H of CH_2OCO, CH-CH_2OCO, and Leu α-CH), 4.42 (1H, m, Met α-CH), 7.15–7.31 (5H, m, aromatic), 8.03 (3H, m, H-CO-N (s at 8.02) superimposed on Pheol NH and Leu NH), 8.22 (1H, s, H-CO-O), 8.31 (1H, d, *J* = 8 Hz, Met NH).– $C_{22}H_{33}N_3O_5S$ (451.6) Calcd. C 58.5 H 7.36 N 9.3 Found C 58.9 H 7.75 N 9.2.

HCO-Met-Leu-Pheol (4)

To a suspension of formate **3** (0.113 g, 0.25 mmol) in dry THF (5 ml) neutral alumina ICN (B IV) (1.5 g) was added. After stirring at room temp. for two days, the alumina was filtered off, washing with EtOAc. Evaporation of the organic solution under reduced pressure gave pure HCO-Met-Leu-Pheol (**4**) (0.101 g, 96%), mp. 157 °C (EtOAc).– $[\alpha]_D$ –61° (*c* 1.0, MeOH).– R_F 0.46 in EtOAc.– IR (KBr): 3284; 1632; 1551 cm^{-1}.– ^{1}H-NMR ([D_6]DMSO): δ (ppm) = 0.81 and 0.85 (6H, two d, *J* = 6.5 Hz, $(CH_3)_2CH$), 1.38 (2H, m, Leu β-CH_2), 1.51 (1H, m, Leu γ-CH), 1.67–1.91 (2H, m, Met β-CH_2), 2.01 (3H, s, SCH_3), 2.39 (2H, t, *J* = 7.5 Hz, CH_2S), 2.62 and 2.82 (2H, A and B of an ABX, *J* = 8, 5.5, and 14 Hz, Ph-CH_2-CH), 3.28 (2H, m, CH_2OH), 3.86 (1H, m, CH-CH_2OH), 4.25 (1H, m, Leu α-CH), 4.40, (1H, m, Met α-CH), 4.76 (1H, t, *J* = 5 Hz, OH), 7.12–7.28 (5H, m, aromatic), 7.71 (1H, d, *J* = 8 Hz, Pheol NH), 8.02 (2H, m, H-CO-N (s at 8.01) superimposed on Leu NH), 8.31 (1H, d, *J* = 7.5 Hz, Met NH).– $C_{21}H_{33}N_3O_4S \cdot 1/2\ H_2O$ (432. 6) Calcd. C 58.3 H 7.92 N 9.7 Found C 58.6 H 8.28 N 9.5.

HCO-Met-Leu-Pheol acetate (5)

A solution of **4** (0.106 g, 0.25 mmol) in Ac_2O (1 ml) and Py (2 ml) was stirred at room temp. overnight. EtOAc was added in excess and the org. layers were washed with chilled saturated aqueous $NaHCO_3$ and water. The org. phases were dried and evaporated under reduced pressure to afford pure title acetate **5** (0.114 g, 98%), mp 172 °C.– $[\alpha]_D$ –46° (*c* 0.5, MeOH).– R_F 0.62 in chloroform-MeOH (9:1).– IR (KBr): 3294; 1736; 1666; 1641; 1551 cm^{-1}.– ^{1}H-NMR ([D_6]DMSO): δ (ppm) = 0.80 and 0.85 (6H, two d, *J* = 6.5 Hz, $(CH_3)_2CH$), 1.36 (2H, m, Leu β-CH_2), 1.52 (1H, m, Leu γ-CH), 1.68–1.89 (2H, m, Met β-CH_2), 1.96 (3H, s, CH_3-CO), 2.00 (3H, s, SCH_3), 2.38 (2H, t, *J* = 7.5 Hz, CH_2S), 2.74 (2H, sharp m, Ph-CH_2-CH), 3.82 and 4.01 (2H, A and B of an ABX, *J* = 6, 4.5, and 11 Hz, CH_2OCO), 4.15 (1H, m, CH-CH_2OCO), 4.23 (1H, m, Leu α-CH), 4.40 (1H, m, Met α-CH), 7.13–7.30 (5H, m, aromatic), 7.92–8.07 (3H, m, H-CO-N (s at 8.00) superimposed on Leu NH and Pheol NH), 8.28 (1H, d, *J* = 7.5 Hz, Met NH).– $C_{23}H_{35}N_3O_5S$ (465.6) Calcd. C 59.3 H 7.58 N 9.0 Found C 59.7 H 7.88 N 8.6.

Cyclization of 4 to 2-oxazoline 6

To a suspension of HCO-Met-Leu-Pheol (**4**) (0.106 g, 0.25 mmol) in dry THF (1 ml), cooled at 0 °C, TEA (0.08 ml, 0.55 mmol) and a solution of MesCl (0.04 ml, 0.5 mmol) in dry THF (1 ml) were added. After stirring at 0 °C for 5 min and at room temperature for 19 h, DBU 96% (0.08 ml, 0.5 mmol) was added. The mixture was refluxed for 3 h and then evaporated under reduced pressure. EtOAc was added in excess and the org. layers were washed with water, dried and evaporated to afford an oil (0.107 g). Purification by PLC (EtOAc as eluant) afforded pure oily 2-oxazoline **6** (0.032 g, 32%).– $[\alpha]_D$ = –34° (*c* 1.0, $CHCl_3$).– R_F 0.35 in ethyl acetate.– IR ($CHCl_3$): 3412; 1672; 1494 cm^{-1}.– ^{1}H-NMR ($CDCl_3$): δ (ppm) = 0.93 (6H, d, *J* = 6 Hz, $(CH_3)_2CH$), 1.50–1.71 (3H, m, $(CH_3)_2CH$-CH_2), 1.96–2.11 (2H, m, Met β-CH_2), 2.09 (3H, s, SCH_3), 2.59 (2H, t, *J* = 7.5 Hz, CH_2S), 2.65 and 3.01 (2H, A and B of an ABX, *J* = 8, 5, and 14 Hz, Ph-CH_2-CH), 4.03 and 4.22 (2H, two apparent t, CH_2O), 4.36 (1H, m, CH-CH_2O), 4.74 (2H, m, Met α-CH and Leu α-CH), 6.89 (2H, m, Leu NH and Met NH), 7.13–7.37 (5H, m, aromatic), 8.18 (1H, s, H-CO-N).

Biological Assay

Cells

Human peripheral blood neutrophils were purified employing the standard techniques of dextran (Pharmacia) sedimentation, centrifugation on Ficoll-Paque (Pharmacia) and hypotonic lysis of red cells. The cells were washed twice and resuspended in KRPG (Krebs-Ringer-phosphate containing 0.1%

w/v glucose, pH 7.4) at a concentration of 50×10^6 cells/ml as described [14]. The percentage of neutrophils was 98–100% pure.

Random Locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Italy) and the migration into the filter was evaluated by the method of leading-front [15]. The actual control random movement is 32 μm ± 3 SE of ten separate experiments done in duplicate.

Chemotaxis

In order to study the potential chemotactic activity, each peptide was added to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution (10^{-2} M in DMSO) with KRPG containing 1 mg/ml of bovine serum albumin (Orha Behringwerke, BRD) and used at concentrations ranging from 10^{-11} M to 10^{-5} M. Data were expressed in terms of chemotactic index, which is the ratio: (migration towards test attractant minus migration towards the buffer)/migration towards the buffer.

Superoxide Anion (O_2^-) Production

O_2^- release was monitored continuously in a thermostatted spectrophotometer as superoxide dismutase-inhibitable reduction of ferricytochrome c (Sigma, USA), as described elsewhere [16]. At zero time, different amounts (10^{-8} – 10^{-5} M) of each peptide were added and the absorbance change accompanying cytochrome c reduction was monitored at 550 nm. Results were expressed as net nmoles of $O_2^-/2 \times 10^6$ cells/5 min. Neutrophils were incubated with 5 μg/ml cytochalasin B (Sigma) for 5 min prior to activation by peptides.

Enzyme Assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity [16]; this was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus* (Sigma). Enzyme release was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 μg/1×10^7 cells/min. To study the degranulation-inducing activity of each peptide, neutrophils were first incubated with cytochalasin B for 15 min at 37 °C and then in the presence of each peptide in a final concentration of $10^{-8} - 10^{-5}$ M for a further 15 min.

Statistical Analysis

The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

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Received: June 1, 1995 [FP025]